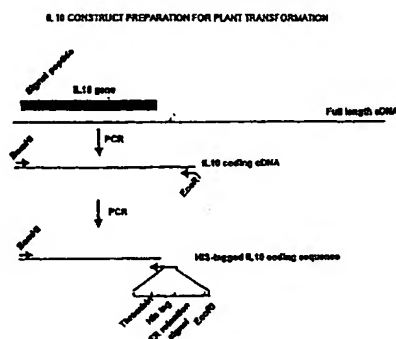




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/82		A2	(11) International Publication Number: WO 99/67401
			(43) International Publication Date: 29 December 1999 (29.12.99)
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(22) International Filing Date: 21 June 1999 (21.06.99)		(74) Agents: SECHLEY, Konrad, A. et al.; Gowling, Strathy & Henderson, Suite 2600, 160 Elgin Street, Ottawa, Ontario K1P 1C3 (CA).	
(30) Priority Data: 09/102,050 22 June 1998 (22.06.98) US		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
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(54) Title: NON-FOOD CROP PLANT BIOREACTOR



(57) Abstract

A novel method for the production of transgenic proteins of interest suitable for oral administration is disclosed. The method involves the preparation of a protein of interest suitable for oral administration within a non-food crop plant comprising, transforming the non-food crop plant with a suitable vector containing a gene of interest and appropriate regulatory regions to ensure expression of the gene of interest within the non-food crop plant, such that the non-food crop plant is characterized as being non-toxic, non-addictive, palatable, and requiring minimal or no processing prior to oral administration. An example of a non-food crop plant is low alkaloid tobacco. Proteins of interest may include pharmaceutically active proteins such as growth regulators, insulin, interferons, interleukins, growth hormone, erythropoietin, G-CSF, GM-CSF, hPG-CSF, M-CSF, Factor VIII, Factor IX, tPA, antibodies, antigens and combinations and/or derivatives thereof.

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NON-FOOD CROP PLANT BIOREACTOR

The present invention relates to the use of a non-food crop plant as a bioreactor. More specifically this invention relates to the expression of transgenes of interest for oral administration using non-food crop plants.

BACKGROUND OF THE INVENTION

Full citations for references appear at the end of the examples section.

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Numerous plants have proven themselves to be amenable to transformation with heterologous genes and for some time tobacco has been the model system for plant transformation. Despite the fact that crop-protection focused biotechnologies have not found application in non-food crop plant production, a major role does remain for such plants as bioreactors. An example of a non-food crop plant is tobacco, which is capable of producing high levels of soluble protein (fraction 1 protein, F1P; Woodleif et al 1981) and pilot systems have been developed to purify this fraction for use as a high protein dietary supplement (Montanari et al 1993).

20

Expression of mammalian genes in several plants including tobacco and *Arabidopsis* has been recognized as efficient, low cost, non-sterile bioreactors for the production of proteins valuable to both medicine and industry (Ma and Hein 1995). Recently evidence was presented that demonstrated that the four chains of the secretory immunoglobulin were properly expressed and assembled in plants and that the antibody was fully functional (Ma et al 1995). Furthermore, bacterial (Haq et al 1995), and viral (Mason et al 1996) antigens produced in transgenic tobacco and potato effectively immunized mice when the transgenic potato was administered orally. However, prior to the administration of plant tissue obtained from transgenic tobacco, the proteins had to be partially purified. Clearly steps involving processing are undesirable if ease of oral administration is to be maximized as well as minimizing any associated costs for production.

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From both a regulatory and public safety stand point non-food crop plants are ideal species for the transgenic production of biologically active proteins. Non-food crop plants minimize the risk of accidental leakage of transgenic plant material expressing genes for biologically active proteins into the human food chain. Other plant bioreactor systems based on canola (Rooijen et al. 1995), potato (Manson et al 1996), rice and cassava (Ma and Hein 1995) do not offer this advantage. Furthermore, non-food crop plants can be selected so that production in areas where there are no naturally occurring wild species further minimizes the risk of gene leakage to the local flora, an example of this would be to grow tobacco in regions where tobacco does not overwinter, such as Canada. With any non-food crop plant, transgenic proteins can be produced using any tissue or organ of the plant. However if protein production is based on leaves, not seeds or tubers, and when coupled with the fact that the leaves are harvested before flowering there is virtually no risk of uncontrolled bioreactor plants occurring in future crop seasons.

15

However, many non-food crop plants contain high levels of secondary plant products making plant tissue obtained from these plants unsuitable for direct oral administration. Earlier studies have described the administration of tobacco-derived proteins to mice, however, the proteins were in a partially purified form. For example the study by Mason et al (1996) involved the direct oral administration of viral antigens expressed in potato tuber and tobacco. The potato tuber samples were directly fed to mice, yet the antigen, when obtained from tobacco, had to be partially purified using sucrose gradients prior to administration to mice.

20

The breeding of low alkaloid-containing tobacco plants has been reported (Chaplin 1977), however, use of such a plant as a bioreactor has not been suggested. Recently, specific alteration of nicotine levels within tobacco, by either over expression (i.e. increasing) or antisense expression (decreasing) of putrescine N-methyltransferase, a rate limiting enzyme involved in the nicotine biosynthetic pathway, has been suggested (U.S. 5,260,205, issued November 9, 1993 and U.S. 5,369,023, issued November 29, 1994; inventors Nakatani and Malik). However, these methods are directed to the

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alteration of nicotine levels so that the levels of other alkaloids that affect the flavours and aroma of tobacco are not modified in any manner. These plants would not be suitable for use as a non-food crop plant as describe herein, since the levels of only a select group of alkaloids have been reduced. No nicotine free plants were actually
5 produced, nor was the use of these transgenically modified tobacco plants, as a bioreactor for the synthesis of proteins of interest, suggested. However, such methods may be used and augmented in order to produce non-food crop plants where the total alkaloid level is reduced. Such an approach may be useful for the production of low alkaloid plants as a bioreactor for the synthesis of proteins of interest as contemplated by this invention.

10

Thus there is a need to provide a non-food crop plant capable of being used to prepare transgenic proteins of interest suitable for oral administration.

SUMMARY OF THE INVENTION

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The present invention relates to the production of a protein of interest using a non-food crop plant.

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According to the present invention there is provided a method for the preparation of a protein of interest suitable for oral administration within a non-food crop plant comprising, transforming the non-food crop plant with a suitable vector containing a gene of interest and appropriate regulatory regions to ensure expression of the gene of interest within the non-food crop plant, such that the non-food crop plant is characterized as being non-toxic, non-addictive, palatable, and requiring little or no processing prior to oral
25 administration.

This invention further relates to the above method wherein the non-food plant is characterized in having a low alkaloid level. This invention also includes methods utilizing a plant characterized in having a low nicotine level.

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This invention also relates to the above method wherein the protein of interest includes pharmaceutically active proteins, such as growth regulators, insulin, interferon and related compounds, interleukins, growth hormone, erythropoietin, G-CSF, GM-CSF, hPG-CSF, M-CSF, Factor VIII, Factor IX, tPA, antibodies, antigens and any combinations and derivatives thereof.

Furthermore, this invention includes the above method wherein a suitable vector contains a gene of interest along with appropriate regulatory regions to ensure expression of the gene of interest within desired plant tissues.

10

This invention also provides for compositions comprising a tissue from a non-food crop containing a transgenic protein of interest.

Another aspect of an embodiment of this invention is a method for the treatment of a medical aliment by administering a suitable amount of the above composition comprising a tissue from a non-food crop plant containing as a pharmaceutically active component a transgenic protein of interest.

This invention is also directed to a method for preparing a pharmacologically active protein within a non-food crop plant comprising;

20

i) selecting a non-transformed tobacco plant comprising from about 0.2% to about 20% of the total alkaloid, and characterized as being non-toxic, non-addictive, and palatable;

25

ii) transforming said non-transformed tobacco plant with a vector comprising a gene capable of encoding said pharmacologically active protein and 5' and 3' regulatory regions to ensure expression of the gene within said non-transformed tobacco plant to produce a transformed plant; and

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- 5 -

- iii) obtaining said pharmacologically active protein from said transformed plant.

Furthermore, this invention embraces a method for the preparation of IL-10
5 comprising;

- i) transforming a plant with a vector comprising a gene encoding IL-10,
operatively linked with 5' and 3' regulatory regions;
- 10 ii) selecting transformed plants that contain IL-10;
- iii) growing and harvesting selected plants; and
- iv) extracting IL-10 from said selected plants.

15

Also included within the present invention is the above method wherein said IL-10 is human IL-10. Optionally, the vector may comprise sequences that enhance expression of said IL-10, such as an ER retention signal. Furthermore, the vector may also encode a proteolytic cleavage site, an affinity tag, or a combination thereof.

20

Although the present invention is exemplified by the preparation of transgenic proteins of interest using tobacco, in practice any non-food crop plant may be used for the preparation of a protein of interest following the methods as disclosed herein.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings wherein:

5

FIGURE 1 shows the oligonucleotide cassettes for integration of TMV 5'-untranslated region and PR-1b transit peptide sequences into transgene constructs. Figure 1(A) the TMV leader sequence. Figure 1(B) the PR-1b signal peptide sequence. Figure 1(C) the amino acid translation of encoded signal peptide. Identity, sequence, and position of each oligonucleotide within the cassettes are indicated. TMV sequence is marked in bold, PR-1b signal peptide sequence in plain letters and additional sequence in italics. Nucleotides constituting all or part of the initiation codon are present within boxes. Oligonucleotides #1091 (SEQ ID NO:1); #1092 (SEQ ID NO:4); and #1093 (SEQ ID NO:2) represent the TMV oligo set. Oligonucleotides #1091, #1092 (SEQ ID NO:1 and 4), and #4361 through #4365 (SEQ ID NOs: 3, 5, 6, 8 and 9 respectively) represent the TMV-PR oligo set.

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FIGURE 2 displays the map of the T-DNA region of pCDX-TL-MAFP11

FIGURE 3 displays the map of the T-DNA region of pMON-TL-MAFP11

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FIGURE 4 is a Western blot showing Type II AFP accumulation in field plants.

Western blot analysis of total soluble protein (2.5 µg) extracts from R1 generation Type II AFP transgenic plants in which the AFP was targeted to accumulate in the extracellular space. Samples are designated by number to indicate the plot from which it was collected and by letter to indicate the transformed parent plant from which the R1 plants were descended. Records at the Delhi station indicate that A plants were descended from parent II4c²-#10, B plants from II4c²-#1, C from pII3a-#7, and D plants were wild-type controls (4D). Total soluble protein extract from a field-grown wild type plant was included in the analysis as a

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negative control and mixed with mature Type II AFP (25 ng) purified from sea raven sera to generate the positive control (AFP).

FIGURE 5 shows the cDNA sequence of human IL-10. The cDNA sequence is denoted in small letters, the encoded protein sequence is denoted in capital letters below the nucleotide sequence with the one letter amino acid code. The signal peptide sequence is boxed and shaded. Oligonucleotides used to amplify hIL-10 sequences or to fuse other sequences to the hIL-10 gene are shown in capital letters underlined by an arrow. Restriction sites introduced by PCR are shaded. Encoded amino acids are shown above the hIL-10-His-KDEL oligonucleotide sequence.

FIGURE 6 shows a schematic depiction of three IL-10 constructs used in plant transformation as described herein. Figure 6 (A) shows the full length, 1601 bp cDNA of IL-10. Transformed plants comprising this construct are designated as "F" plants in Figure 7. Figure 6 (B) shows a truncated IL-10 sequence comprising nucleotides 4-732, with only a portion of the 3' UTR (168 bp). Transformed plants comprising this construct are designated as "E" plants in Figure 7. Figure 6 (C) shows an IL-10 construct with the 3' UTR portion removed, and a thrombin recognition sequence, a His tag and a KDEL sequence added to the 3' end of the IL-10 sequence. The region containing the thrombin-His tag and KDEL sequence is magnified to show the order of those sequences with respect to the hIL-10 gene. Transformed plants comprising this construct are designated as "G" plants in Figure 7. The hIL-10 coding region is depicted by a dark shaded box, the signal peptide is shown by a light shaded box, and the cDNA is shown by a straight line. Oligonucleotides are represented by short arrows.

FIGURE 7 shows the IL-10 protein levels within transgenic plants as determined by ELISA. The protein concentration was determined and compared against a hIL-10 standard curve. Individual transformed plants were analyzed. Plants denoted

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“E” were transformed with hIL-10 construct containing coding sequences and a short 3' UTR; plants denoted “F” were transformed with hIL-10 construct containing coding sequences and the complete 3' UTR; plants denoted “G” were transformed with hIL-10 construct containing coding sequences fused to thrombin-His tag-KDEL sequence.

5

DESCRIPTION OF PREFERRED EMBODIMENT

From both a regulatory and public safety stand point non-food crop plants are ideal species for the transgenic production of biologically active proteins since the risk of leakage of transgenic plant material into the human food chain is negligible. Other plant bioreactor systems based on food crops do not offer this advantage. Even though non-food crop plants are ideal species for use as bioreactors from both a regulatory and public safety point of view, there are several obstacles that must be overcome prior to their use as bioreactors.

10

A major problem with the use of non-food crop plants, for example tobacco, as bioreactors is that these plants may contain undesirable secondary plant products. Secondary plant products are constituents that are generally toxic or reduce the palatability of the plant tissue or that are addictive in nature. This is a significant concern since one of the benefits of preparing proteins of interest within a plant is that large quantities of protein may be required for administration (Ma and Hein, 1995), therefore any toxic, addictive, or otherwise non-desirable products should be avoided within the plant tissue. For example, tobacco plants contain high levels of secondary plant products such as nicotine and related alkaloids, making the plant tissue unsuitable for the direct oral administration. Earlier studies have described the administration of tobacco-derived proteins to mice, however, the proteins were in a partially purified form. For example, the study by Mason et al (1996) involved the direct oral administration of viral antigens expressed in potato tuber and tobacco. The potato tuber samples were directly fed to mice, yet the antigen, when obtained from tobacco, had to be partially purified using sucrose gradients prior to administration to mice. It is desirable to prepare transgenic proteins within non-food crop plants that permit the direct use of plant tissue for oral administration, or following minimal processing.

By "non-food crop plant" it is meant a plant that has traditionally not been used, or grown for food purposes. Examples of non-food crop plants include, but are not limited to, ornamental plants, tobacco, hems, weeds, and the like and trees. It is

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contemplated that such non-food crops plants may be used for the production of a protein of interest. In order to that the non-food crop plant to be used as a bioreactor for the production of a transgenic-expressed protein of interest suitable for direct oral administration; or requiring only minimal processing prior to oral administration, such a non-food crop plant is characterized as being:

- a) is non-toxic;
- b) is palatable following minimal or no processing; and
- c) is characterized in having low levels of non-desirable secondary plant products such as alkaloids, nicotine, or the like.

10

However, if it is desired that the protein of interest is to not be orally administered, than any non-food crop plant may be used for the production of a protein of interest following the methods of the present invention as described herein. In such applications where there is no need for orally administering the protein of interest, the protein of interest may be combined with other nucleic acid sequences that are useful for the purification of the protein of interest in order that the protein be suitable for further use.

Use of the terms "low nicotine" and "low alkaloid" with reference to plants, such as, but not limited to tobacco, means a plant that contains a significantly lower concentration of alkaloids when compared with a similar plant comprising regular alkaloid content. For example, which is not to be considered limiting in any manner, a low alkaloid tobacco plant may be defined as a plant that contains less than from about 0.2% to about 70 % of the total alkaloids present in plants comprising a regular level of alkaloids. More preferably a low-alkaloid plant is one that contains about 0.2% to about 10% of the total alkaloids present in plants comprising a regular level of alkaloids. An example of a low alkaloid plant, that comprises about 2.8% of the total alkaloid level of a regular tobacco, is 81V-9 (see Table 1, Example 4). Similarly, a low nicotine tobacco plant is one that contains a significantly lower concentration of nicotine when compared with a similar plant comprising regular nicotine content. For example, which is not to be considered limiting in any manner, a low nicotine tobacco plant may be defined as a

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plant that contains less than from about 0.2% to about 70 % of the total nicotine present in plants comprising a regular level of nicotine. More preferably a low-nicotine plant is one that contains from about 0.2% to about 10% of the total nicotine present in plants comprising a regular level of nicotine. An example of a low nicotine plant, that
5 comprises about 2.6% of the total nicotine level of regular tobacco, is 81V-9 (Table 1, Example 4).

Such low alkaloid or low nicotine plants may be obtained through conventional breeding programs (e.g. Chaplin, 1977), through selective down regulation of undesired
10 genes (e.g. U.S. 5,260,205, issued November 9, 1993 and U.S. 5,369,023, issued November 29, 1994; inventors Nakatani and Malik), or by any other means e.g. mutagenesis followed by selection of desired traits. However, as is known to one of skill in the art, the ultimate nicotine or alkaloid level may still depend upon the environment under which the plant is grown.

15

By "minimal processing" it is meant the rapid processing of transgenic proteins produced within non-food crop plants. Such rapid processing could involve, but is not limited to, methods as those employed for the preparation of F1P as disclosed in Woodleif et al (1981). These include the aqueous extraction of soluble protein from
20 green tobacco leaves by precipitation with KHSO_4 , following removal of chloroplastic debris. Other methods may include large scale maceration and juice extraction in order to permit the direct use of the extract. If mechanically extracted at the field site in the presence of water, this extract may be further processed prior to administration. Protein compositions prepared from minimally processed plant tissues comprise the transgenic
25 protein of interest along within plant-derived components including cellular components and other soluble materials.

By "suitable vector" it is meant a vector comprising a gene of interest that is capable of being expressed within plant tissue. Such a vector may also include
30 ubiquitous or tissue specific promoters and other 5' and 3' regulatory elements as would be known to one of skill in the art. Other elements that may be included with this vector

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include sequences for targeting the protein of interest to the cytosol or secretory pathway such as, but not limited to, the C-terminal KDEL sequence, an endoplasmic reticulum retention motif (Schouten et al 1966). Other retention signals, as would be known to one of skill in the art, may also be used for this purpose. Furthermore, such a vector may
5 include marker genes for the detection of expression within the transgenic plant, and other sequences that aid in the purification of a protein of interest encoded by the gene of interest, including proteolytic cleavage, column chromatography and the like. An example, which is not to be considered limiting in any manner, of a sequence that aids in the purification of a protein of interest includes an affinity tag, for example, sequences
10 that encode a His tag. However, it is to be understood that other affinity tags, as are known within the art, may also be used for the purpose of purification. An example, which is not to be considered limiting in any manner of a sequence that aids in proteolytic cleavage, and is used to separate the protein of interest from other translated sequences such as a KDEL sequence includes, for example, a sequence encoding a thrombin
15 cleavage site. Again, it is to be understood that other proteolytic cleavage sites may also be used as would be evident to one of skill in the art.

By "protein of interest" it is meant any protein that is to be expressed in a transformed plant. Such proteins may include, but are not limited to, pharmaceutically
20 active proteins, for example IL-1, IL-2, IL-3 ... IL-12, EPO, CSF, including G-CSF, GM-CSF, hPGCSF, M-CSF, Factor VIII, Factor IX, tPA, hGH, receptors, receptor agonists, antibodies, neuropolypeptides, insulin, vaccines, growth factors, growth regulators, antigens, their derivatives and the like.

25 By "oral administration" it is meant the administration of a tissue or organ of a non-food crop plant, for example the leaf, root, fruit etc with minimal or no prior processing. Such tissues or organs may be provided in the form of a salad or the like along with other pharmaceutical ingredients, or ingredients to increase palatability, if desired. It is also contemplated that minimal processing of the tissue or organ may take
30 place prior to administration of the extract. For example, proteins secreted within the extracellular space of leaf tissues could be readily obtained using vacuum or centrifugal

extraction, or tissues could be extracted under pressure by passage through rollers or grinding or the like to squeeze or liberate the protein free from within the extracellular space. Minimal processing could also involve preparation of crude extracts of soluble proteins, since these preparations would have negligible contamination from secondary
5 plant products.

By "medical aliment" it is meant a defined medical condition that can be treated a specific pharmaceutical suitable for the treatment of the condition. Examples of such medical ailments and their corresponding suitable pharmaceutical include, but are not
10 limited to: diabetes and the administration of IL-4 or insulin or a combination thereof; conditions related to blood clotting and the administration of Factor VIII, Factor IX or tPA or combinations thereof; medical conditions requiring the stimulation of progenitor cells to monocytes/macrophages and the administration of G-CSF, GM-CSF, hPG-CSF, M-CSF or combinations thereof; viral infections and the administration of interferons e.g.
15 interferon- α , interferon- β , interferon- τ .

Several methods have been proposed for the removal of nicotine from tobacco, however, these processes typically involve the treatment of post harvest-tissue. For example the use of solvents (EP 10,665, published May 14, 1980; inventors Kurzhalz and
20 Hubert), or potassium metabisulphite, potassium sulphate and nitrate (U.S. 4,183,364, issued January 8, 1980; inventor Gumushan) have been proposed as methods for removing nicotine from tobacco leaves. All of these treatments are designed to maintain the flavour and aroma of the tobacco, and these methods involve extensive post-harvest processing and are therefore not suitable for the preparation of products as described in
25 this invention.

Specific alteration of nicotine levels within tobacco, by either over expression (i.e. increasing) or antisense expression (decreasing) of putrescine N-methyltransferase, a rate limiting enzyme involved in the nicotine biosynthetic pathway, has been suggested (U.S.
30 5,260,205, issued November 9, 1993 and U.S. 5,369,023, issued November 29, 1994; inventors Nakatani and Malik). These methods are directed to the alteration of nicotine

levels so that the levels of other alkaloids that affect the flavours and aroma of tobacco are not modified in any manner. No nicotine free plants were actually produced, nor was the use of these transgenically modified tobacco plants, as a bioreactor for the synthesis of proteins of interest, suggested. However, such methods, if modified to reduce total
5 plant alkaloid levels, may be used in order to produce non-food crop plants wherein total alkaloid levels are reduced.

In order to establish the efficacy of transgene expression in a low alkaloid plant, for example a nicotine-free tobacco such as but not limited to 81 V-9, and ensure soluble
10 protein production, several proteins of interest were expressed within a low alkaloid plant. These proteins include IL-4, IL-10, and a Type II antifreeze protein (AFP), however, it is to be understood that other proteins of interest may be also used to transform a low alkaloid, or low nicotine plant according to the methods of this invention.

15 IL-4 is a growth and differentiation factor for T-cells. In particular, IL-4 promotes the development of the subset of T helper (Th2) cells from native T cells upon antigen stimulation. IL-4 producing Th2 cells generally protect against the onset of many organ-specific autoimmune diseases, including type 1 diabetes. Thus the availability of
20 an abundant source of IL-4 may prove invaluable for the immunotherapy of diabetes. Transgenic low-nicotine tobacco plants can be used in direct feeding studies to determine the efficacy of the recombinantly produced protein.

Crohn's disease is a chronic inflammatory bowel disease with frequent remissions
25 and exacerbations, and several extra-GI manifestations. Lesions are typically discontinuous and can occur anywhere within the GI tract. The most common locations are ileum and colon, and formation of strictures and fistulas are common. In contrast, ulcerative colitis is contiguous and characteristically affects the colon. While the etiology of both these inflammatory bowel diseases remain unknown, there is data demonstrating
30 the participation of immune cells in these conditions. Cytokines may direct the intensity, extent, and eventual outcome of inflammatory lesions. Interestingly, mice made deficient

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for IL-10; by targeted gene disruption, develop bowel inflammation and lesions resembling inflammatory bowel disease, which can be improved by systemic or topical administration of IL-10 (Kuhn et al. 1993). The balance between proinflammatory cytokines such as interleukin-1, interleukin-6, γ -interferon and tumor necrosis factor and
5 anti-inflammatory cytokines such as receptor antagonists, IL-10, IL -4, and transforming growth factor beta (TGF β) might ultimately determine the outcome of inflammatory bowel disease (Fiocchi 1993) . By its nature in down regulating pro-inflammatory cytokines, and up regulation of interleukin-1 receptor antagonists, IL-10 may be an effective strategy to treat inflammatory bowel disease. The present treatments for
10 inflammatory bowel disease are empirically designed to reduce inflammation and rely heavily on corticosteroids and immuno-solisolates (5-ASA).

Administration of cytokines orally may offer an alternative to systemic application or topical application and may have local and systemic effects. Interestingly
15 acid inactivation and protease digestion does not appear to inactivate all cytokine effects, in the limited experience to date using an oral approach. Bioactivity may be retained even in the presence of trypsin and chymotrypsin, at least for IL-6, suggesting heavy glycosylation and other factors confer acids stability to cytokines (Rollwagen and Baqar 1996). Furthermore, interferons can suppress collagen induced arthritis in rats when given
20 in high dose orally. Oral cytokines may simplify an approach to delivery and could be useful in modele of infection, HIV, autoimmunity and transplantation rejection.

Human IL10 has also been cloned, and both mouse and human cDNA encode functionally and structurally similar proteins (with 178 amino acids) including a
25 hydrophobic leader sequence and greater than 70% homology in predicted amino acid sequences.

In addition to production by CD4+ TH-2 clones, IL-10 is produced by B cells, activated mast cells, macrophages, monocytes and keratinocytes. There are multiple roles
30 for IL-10 in the regulation of the immune response, including inhibition of T cell, monocyte and macrophage function, as well as inhibition of cytokines particularly those

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of the TH-1 T cell subset (interferon gamma) and inhibition of B cell proliferation. The inhibition of various cytokines such as interferon gamma, GM-CSF and TNF following lectin or anti-CD3 activation is both transcriptional and translational. There is also inhibitory activity on MHC class II antigen expression, which is a protein central to the recognition of antigen presenting cells by T-cell. IL-10 also inhibits the production of hydrogen peroxide and nitric oxide following interferon-gamma activation. Therefore there is a strong basis for the concept that IL-10 naturally occurs to provide negative regulation to immune responses.

Gene constructs were transformed into *N. tabacum* cv. Xanthi and 81V9-4 tobacco strains (available from Agriculture and Agri-Food Canada, Pest Management Research Center, Delhi Farm, Genetics Section). 81V9-4 is a flue-cured tobacco line containing only trace amounts of alkaloids and is an ideal component for tobacco-based molecular farming applications. Gene constructs were built for production of pro and mature forms of a Type II antifreeze protein (AFP) or IL-4, to be accumulated in the cell cytosol and extracellular space. Several different constructs comprising IL-10 in order to optimize expression of the gene within a non-food crop plant were also examined.

IgG expression in plants lends support to the idea that passage through the endoplasmic reticulum may enhance protein accumulation. In plants bred to simultaneously express mouse IgG light and heavy chain proteins targeted to the extracellular space, the amount of each protein increased up to 60-fold over that seen when expressed individually into the same compartment (Hiatt et al 1989). Expression of both protein components into the cytosol, however, did not affect their level of accumulation. IgG processing and assembly in lymphocytes occurs through the action of heavy-chain binding proteins present in the endoplasmic reticulum (ER). The observed increase in yield when both proteins were targeted to the extracellular space has been attributed to an enhanced stability contingent on IgG assembly. Consistent with this hypothesis, active antibody complexes were observed when the proteins were targeted to the extracellular space but not when targeted to the cytosol.

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To further maximize expression levels and transgene protein production the gene encoding the protein of interest, the codon usage within the non-crop plant of interest should be determined. Also, it has been shown that endoplasmic reticulum retention signals can dramatically increase transgene protein levels, for example the KDEL motif (Schouten et al 1996). Replacing any secretory signal sequence with a plant secretory signal will also ensure targeting to the endoplasmic reticulum (Denecke et al 1990).

10 Examples

Gene Construction

To maximize potential AFP production at the translational level, the native 5'-untranslated region of the fish cDNA was replaced with the 5'-untranslated leader region of the tobacco mosaic virus (TMV) (Richards et al 1977 & 1978, Sleat et al 1988). The fish signal peptide sequence was also replaced with that of the tobacco pathogenesis-related protein 1b (PR-1b) (Cornelissen et al 1986, Sijmons et al 1990, Denecke et al 1990). Oligonucleotide cassettes were designed: three oligonucleotides, #1091, (SEQ ID NO:1); #1092 (SEQ ID NO:4); and #1309 (SEQ ID NO:2), which would hybridize to generate the complete TMV leader sequence (TMV Cassette: Figure 1A); and five oligonucleotides #4361-#4365 (SEQ ID NOs: 3, 5, 6, 8 and 9, respectively) which in concert with oligonucleotides #1091 and #1092 (SEQ ID NO:1 and 4) would hybridize to generate a linked TMV leader and PR-1b signal peptide DNA sequence (TMV-PR Cassette: Figure 1B). The 5' end of these cassettes corresponded to a *Bam*H I sticky end restriction site. The 3' end of the TMV cassette was blunt and incorporated an additional adenine nucleotide intended to form the first nucleotide of the start codon while the 3' end of the TMV-PR cassette was compatible with *Nde* I-cut DNA but would not be able to re-generate the restriction site following ligation.

To facilitate addition of the TMV and TMV-PR cassettes, sea raven Type II AFP cDNA was site-specifically mutated according to the method described by Kunkel (1985) to incorporate an *Nde* I restriction site at either bp 157 or 207. These mutations also introduced appropriate in-frame methionine start codons at the junctions between sequences encoding pre and pro, or pro and mature portions of the AFP. To build the gene construct for accumulation of AFP into the cytosol, plasmids containing the mutated Type II AFP cDNAs were initially cut with *Nde*I and made blunt-ended with mung bean nuclease. The cDNA fragments were released by cleavage with *Sal* I, were isolated and directionally ligated into a *Bam*H I/*Sal* I-cut pTZ18 vector together with annealed TMV oligonucleotides #1091, #1092 and #1309. The TMV-proAFP and TMV-mAFP constructs were then cut with *Bam*H I and *Hinc* II, and the isolated AFP gene fragments ligated separately into pMON 893. For gene constructs targeting AFP to the extracellular space, the mutated Type II AFP cDNAs were initially subcloned from their pTZ 19 vectors into *Hind* III/*Sal* I-cut pBluescript vectors to position a *Bam*H I restriction site 5' to the AFP coding sequence. The pBluescript-AFP clones were cut with *Bam*H I and *Nde* I restriction enzymes to remove the sea raven signal peptide sequence or signal peptide and pro-region DNA. The plasmid portions of these digests were isolated and ligated with annealed oligonucleotides #1091, #1092, and #4361 through #4365. The gene constructs were then cut with *Xba* I and *Kpn* I, and separately cloned into pCDX-1.

Cloning of the Type II AFP gene constructs into pMON 893 or pCDX-1 oriented the gene cassette into the vector between the double CaMV 35S promoter (Kay et al 1987) and the NOS polyadenylation sequence. AFP encoded by the transgene constructs was identical to the pro and mature AFP forms present in fish with the exception of one additional methionine at the N-terminal end of the proteins. The T-DNA portion of the final constructs are depicted in Figures 2 and 3. Cleavage of the signal peptide components from the expressed AFPs was predicted (von Heijne 1986) to occur immediately prior to the added methionine in both the pro and mature AFP gene constructs so the AFP accumulating in the cytosol and extracellular space should be identical.

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Gene constructs were transformed into *N. tabacum* cv. Xanthi and 81V9-4 tobacco strains according to the method described by Horsch et al (1988). Attempts to generate plants carrying transgenes for cytosolic accumulation of the AFP were made intermittently over a period of several years. Discs infected with *A. tumefaciens* carrying these gene constructs showed little tendency to form callus and seemed more subject to bacterial infection than comparably treated discs transformed with other transgene constructs. Eventually six transgenic plants were regenerated: one proAFP transgenic in each tobacco strain, three mature AFP transgenics in Xanthi and one mature AFP transgenic in 81V9-4. By contrast, within six months, eight transgenic plants were regenerated carrying gene constructs for AFP accumulation into the extracellular space: two proAFP transgenics in each strain and four mature AFP transgenics in 81V-9. Transformed and regenerated plants were initially selected for kanamycin resistance. Transgenic status was subsequently determined by direct PCR screening for the AFP transgene using primers #3339 5'-TATTTTTTACAACAATTACCAACAAC-3' (SEQ ID NO:10) and #4708 5'-CAGCAGTCATCTGCATACAGCAC-3' (SEQ ID NO:11) which hybridize to the TMV leader and at the 3' end of the sea raven AFP coding sequence, respectively. Type II AFP gene constructs were amplified in 30 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 55°C, and elongation for 2 min at 72°C. This generated amplification products of 440 and 388 bp from the gene constructs for cytosolic accumulation of the pro and mature AFPs, and 532 and 480 bp fragments from gene constructs designed for accumulation of the same AFPs into the extracellular space.

Analysis of Transgene Expression

Plants were removed from the growth chamber and maintained at room temperature under a grow light for a minimum 48 h prior to RNA or protein extraction. RNA was prepared by selective precipitation according to the method of Palmiter (1974). Total RNA (40 µg) was analysed by Northern blots according to the method of Lehrach et al (1977) and probed with [α -³²P]-labelled sea raven AFP cDNA sequence.

Total soluble protein extracts were prepared from three to four leaves taken from the upper half of a healthy plant according to the method described by Gengenheimer (1990). Extracts were dialysed at 4°C against 0.1%, 0.01% and 0.001% ascorbic acid in SpectraPor® #3 dialysis tubing (MWCO 3.5 kDa) and centrifuged for 15 min at 12,000xg to remove precipitate formed during dialysis prior to lyophilization. Samples were resuspended in Millipore®-filtered water and their protein concentrations determined by Bradford assay (16) relative to a BSA standard.

To extract protein present in the apoplast by vacuum infiltration (Sijmones et al 1990), leaves were cut longitudinally into 4 cm x 0.8 cm strips, and rolled lengthwise in 5 cm x 1.8 cm strips of Parafilm® so that the bottom of the leaf was exposed to view. The leaf strip was exposed to vacuum derived from a water aspirator twice for 2 min or until the exposed leaf surface was dark green. Each treated leaf strip yielded 10 ± 2.5 ul of extract. Larger volume samples were too dilute for use and were discarded. Samples which contained green pellets were also discarded to reduce the possibility of cytosolic protein contamination. All remaining extracts from a given plant were pooled and the amount of protein present determined by Bradford (1976) protein assay relative to a BSA standard. Extract concentrations were generally between 0.1-0.2 µg/µl.

20 Western Analysis

Lyophilized protein was resuspended directly in loading buffer (60 mM Tris-HCl, pH 6.8/10% glycerol/5% β-mercaptoethanol/2% (w/v) SDS/ $1.3 \times 10^{-3}\%$ (w/v) bromophenol blue), and were electrophoresed through a 17% polyacrylamide/0.1 M sodium phosphate, pH 6.8/4 M Urea/0.1% SDS gel using 0.1 M sodium phosphate, pH 6.8/0.1% SDS running buffer. Electrophoresis was conducted at 50 V for 3-4 h or until the 6 kDa pre-stained marker was at the bottom of the gel. Western blots were prepared according to the method described by Burnette et al (1981) and probed with polyclonal antibody to the mature sea raven AFP. A chemiluminescence detection kit (Amersham) for probing of Western blots was used to detect bound first antibody. All reactions were carried out according to the manufacturer's directions.

Example 1: AFP Gene Expression

Protein Accumulation

5 Western blot analysis of total soluble protein extracted from plants carrying genes for cytosolic AFP expression did not find evidence of either pro or mature AFP accumulation even when up to 80 µg of protein extract were analysed. In contrast, a protein which co-migrated with mature AFP from sea raven and cross-reacted with antibody to Type II AFP was detected in as little as 2.5 µg total soluble protein extract
10 of plants in which the AFP was targeted to the extracellular space (Figure 4). This protein was unique to the transgenic plants and was absent in extracts of a wild-type plant and a plant, pII3a-#9, which had been transformed and regenerated but determined to be non-transgenic by PCR. Insufficient resolution was obtained with these extracts to differentiate size differences between the AFPs produced by the plants carrying
15 transgenes for production of pro or mature AFPs. This was probably due to the viscosity of the total soluble protein extracts which often caused distortion of samples during the running of gels. For this reason, positive control Type II AFP was mixed with total soluble protein extract from a lab-grown wild-type plant to generate a suitable size standard. Wild-type protein extract was not mixed with the molecular weight markers,
20 so the size of plant-produced AFP could not accurately estimated from these extracts. AFP accumulation varied between plants and appeared slightly higher in plants II4c²-#1 and 10 which carried genes for mature AFP production. Based on Western blot comparison with a known amount of Type II AFP, the amount of AFP produced by the plants has been estimated to be approximately 0.5-1% of the total soluble protein.

25

mRNA Transcription from Transgenes for Cytosolic AFP Accumulation

RNA extracts of plants transgenic with gene constructs for cytosolic AFP accumulation were analysed by Northern blot to determine if the AFP transgene was
30 transcribed. Two plants, Xanthi TmSR #1 and 81V9-4 TmSR #1, carrying transgenes for AFP accumulation into the cell cytosol were found to produce a 0.96 kb RNA

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transcript which hybridized with the AFP cDNA used as a probe: on longer exposure of the blots, the same RNA transcript was also seen in extract from Xanthi TmSR #2 and slightly larger one of approximately 1.02 kb was detected in extract from 81V9-4 TpSR #3. Transcripts from pro and mature AFP transgene constructs were expected to differ by 51 nucleotides. Both plant-produced AFP gene transcripts were both considerably larger than a PCR-generated control sample estimated to be 0.36 kb and representing the size of the mature AFP transgene between the TMV leader and the 3' end of the coding sequence. This was consistent with the size difference expected based on the additional coding sequence and 3'-untranslated region (155 nt total) present in the transgene construct, and with the use of the NOS polyadenylation sequence to obtain polyadenylated transcripts. Both transcripts were smaller than an equivalent gene transcript of 1.06 kb produced in a plant carrying the transgene for targeted mature AFP accumulation into the extracellular space. This size difference was consistent with the addition of the 90 nt signal peptide coding sequence to the transgene construct for secretion of the AFP. Based on EtBr staining, more RNA was loaded onto the gel from the plant producing AFP for accumulation into the extracellular space compared to the amount of RNA from the plants targeting the AFP to the cytosol. No comparison could be made regarding the relative amounts of AFP mRNA transcribed from these constructs. However, similar loadings were achieved between the plants targeting the AFP to the cytosol. The difference in amount of transcript observed, therefore, suggests that this transgene construct was being expressed at variable levels in the different plants.

AFP Present in the Apoplast

Vacuum infiltration extracts were prepared from AFP-producing plants to determine if the expressed AFP was present in the extracellular space. These extracts were not subject to the viscosity and sample distortion associated with total soluble protein extracts, so were also considered more suitable to estimate the sizes of the plant AFPs. Western blot analysis confirmed the presence of AFP. AFP was detected in plants pII3a-#7 and II4c²-#1 which carried mature and proAFP transgenes respectively, as a diffuse band of approximately 14.7 kDa which migrated at a similar rate to mature AFP

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isolated from fish sera. A small mobility difference was observed between the AFPs produced by the two plants, however, proAFP isolated from fish sera was distinctly larger than either plant-produced AFP.

5 Having established the presence of AFP in vacuum infiltration extracts of those plants carrying transgenes for extracellular AFP accumulation, the extracts were concentrated ten-fold and tested for thermal hysteresis activity and effects on ice crystal morphology using the nanolitre osmometer. Extracts from plants expressing gene constructs for either the pro or mature AFP both showed evidence of AFP activity. Ice
10 crystals grown in these plant extracts were seen as the characteristic eye-shaped bipyramids produced in the presence of native Type II AFP. Thermal hysteresis activity was measured and based on comparison with a standard activity curve the amount of active AFP present in the extracellular space was estimated as 2% of the total protein present in the compartment.

15 RNA analysis of the transgenic plants has shown that they are able to transcribe the integrated transgene constructs, and that the size of the plant AFP mRNA from both construct types is consistent with that expected for a full-length, polyadenylated transcript. mRNA produced from the transgene for AFP accumulation into the
20 extracellular space was obviously translatable.

 The presence of plant-produced AFPs in vacuum infiltration extracts indicates that the plant recognized the PR-1b signal peptide component and correctly targeted the attached AFPs to the extracellular space. The predicted size of the AFPs for export to the
25 extracellular space is 18.7 or 17 kDa with the signal peptide component attached and 15.8 or 14.1 kDa without the signal peptide for the pro and mature AFPs, respectively. Western blot analysis of the plant vacuum infiltration extracts showed that the AFPs produced from the proAFP and mature AFP transgene constructs and present in the extracellular space both co-migrated with mature Type II AFP and were approximately
30 14.7 kDa. This suggests that not only is the plant capable of cleaving the signal peptide from the expressed AFP but that it can remove most or all of the pro-region of the AFP.

Therefore, this example demonstrates that a low alkaloid plant is capable of expressing an immunologically detectable protein of interest that retains its native activity.

5 **Example 2: Interleukin-4 expression**

Full length murine IL-4 cDNA is 585 base pairs long (Lee et al 1988) and codes for a 140 amino acid preprotein with a 40 amino acid secretory signal (Otsuka et al 1987). This gene sequence encoding the 40 amino acid secretory signal was replaced with a
10 plant secretory signal obtained from the tobacco PR1-b secretory signal (Denecke et al 1990) to ensure targeting to the endoplasmic reticulum. The resultant vector was introduced into low-nicotine tobacco plants using the methods described above.

In order to determine the biological activity of plant recombinant IL-4, *in vitro*,
15 the stimulation of growth of murine IL-4 dependent CT.4S cell line (Rapoport et al 1993) is determined. Appropriate dilutions of purified plant recombinant IL-4 is added to cultures containing 5×10^3 CT.4S cells in flat bottom 96-well plates in a final volume of 100 μ l for 48 hr. CT.4S cell proliferation is assessed by addition of 1 mCi/well of [3 H]thymidine 18 hr before termination of culture, and [3 H] thymidine incorporation is
20 determined by liquid scintillation counting.

The immunoreactivity of plant recombinant IL-4 is determined by solid phase sandwich ELISA (Abrams et al 1992). Briefly, the BVD4-1D11 anti-IL-4 mAB is used as a capture antibody and is paired with the biotinylated BVD6-24G2 antiIL-4 mAB as
25 the detecting antibody. Mouse rIL-4 (Pharmingen) is used as a standard.

In vivo activity of plant produced IL-4 is determined in NOD mice. NOD and control strain (insulinitis- and diabetes-free) female mice (20 mice/group) are fed either transgenic low nicotine tobacco leaves expressing recombinantly produced IL-4. At
30 various times during the 4 week treatment, circulating serum levels of plant recombinant IL-4-fed and un-fed NOD and control mice are analyzed by ELISA as described above.

Both NOD and control mice are monitored for any potential toxic effects arising from the plant recombinant IL-4 feeding, and their serum concentrations of IgE and IgG quantified by ELISA. Increase in serum IgE levels indicate that recipient mice are more susceptible to allergic responses.

5

Feeding of plant recombinant IL-4 to protect against the onset of insulinitis and/or Type 1 diabetes in NOD mice is also determined. Neonatal female NOD mice (2-3 weeks of age) fed or not fed with plant recombinant IL-4 are maintained in a specific pathogen free animal facility. Mice are monitored weekly for their blood glucose levels (BGL), and mice that are hyperglycemic (i.e. BGL > 11.1 mmol/L) for two consecutive weeks are diagnosed as Type 1 diabetic. The onset of insulinitis is monitored by immunohistochemical analysis of pancreatic tissue by sacrificing mice at various times (2 weeks to 2 months) after plant recombinant IL-4 feeding is initiated.

15 **Example 3: Interleukin-10 (IL-10) expression**

Full length mRNA encoding human IL-10 (hIL-10) is 1601 nucleotides long, with a short 5' UTR of 30 nucleotides (nt) and a long 3' UTR of 1037 nt. The hIL-10 coding sequence is 534 base pairs long and codes for a 178 amino acid preprotein with a 18 amino acid secretory signal (Vieira et al., 1991). The full length human IL10 cDNA is isolated from polyA RNA by RT-PCR and fully sequenced to ensure fidelity. In order to maximize expression levels and transgene protein production, the IL10 cDNA is examined for codon usage patterns and optimized as necessary (Perlak et al. 1991).

25 Since ER retention signals can increase the concentration of disulfide bonded transgene proteins, therefore the KDEL ER retention motif may be added to the IL-10 cDNA using site directed mutagenesis. For comparative purposes an identical synthetic gene, without the KDEL signal, is prepared and used in the transformation studies (SEQ ID NO:12).

30

The expression system adopts a duplicated 35S enhancer-promoter plus AMV leader sequence (Kay et al. 1987; Jobling and Gehrke 1987). However other constitutive promoters may also be employed, for example, Agriculture and Agrifood Canadas T1276 constitutive promoter from tobacco (see copending application US application serial
5 No.08/593,121). The IL-10 gene is cloned into a T-DNA vector between the T-DNA border sequences using the Bin19 derived plant expression vector pCamTerX. The synthetic IL-10 gene is transformed to tobacco using *Agrobacterium* mediated transformation (Horsch et al. 1989). The *Agrobacterium* strain used is EHA101 carrying the disarmed Ti plasmid pEHA104. The plant selectable marker is neomycin
10 phosphotransferase.

To maximize transgene expression and ensure stable field performance a large number of primary transformants need to be generated, selected for expression levels, screened for copy number, and single-copy high-expressing lines evaluated for field
15 performance. Standard methods (e.g. Northern and Western blotting) are used to evaluate expression levels (Sambrook et al. 1989). ELISA is used to quantify transgene protein yields.

A sequence containing the hIL-10 open reading frame and a truncated 3' UTR (nt
20 4-732) is amplified by PCR using oligonucleotides hIL-10-5'-BamHI and hIL-10-3'-EcoRI (Figure 5 (B)); transformed plants comprising this construct are denoted "E" in Figure 7). The 5' oligonucleotide is designed with a BamHI site at the 5' end, and the 3' oligo is designed with an EcoRI site at the 3' end. These restriction sites enable the easy directional cloning of the construct in the phagemid pBluescript (Stratagene) and in the
25 Bin 19-derived binary vector pCaMter X (Frisch et al., 1995). To determine the effect of the 3' UTR on expression of hIL-10 in plants, the complete cDNA (4-1601; Figure 5 (A)); transformed plants comprising this construct are denoted "F" in Figure 7) is also cloned into pBluescript and pCaMter X.

30 To maximize protein accumulation in the plant cell, the endoplasmic reticulum (ER) retention signal, KDEL (Schouten et al., 1996), is added to the 3' end of hIL-10

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coding sequence; transformed plants comprising this construct are denoted "G" in Figure 7). A His tag is also added before the KDEL signal to facilitate purification of the transgenic protein (see Figure 5 (C), and SEQ ID NO:12, nucleotides 544-630). A thrombin recognition sequence is inserted immediately preceding the His tag that allows the cleavage of the His tag and the KDEL peptides. This third construct encodes a preprotein of 197 amino acids. A single oligonucleotide is used to fuse these sequences to the end of hIL-10 reading frame (see Figures 5 and 6). This oligonucleotide is used in conjunction with oligonucleotide hIL-10-5'-BamHI in a PCR to generate the fragment which was then cloned both in pBluescript and in pCaMter X.

10

Cloning of the IL-10 gene constructs into pCaMter X orients the gene between the double CaMV double 35S promoter (Kay et al., 1987) and the NOS (Nopaline synthase) polyadenylation sequence (Frisch et al., 1995). The plant recombinant IL-10 (prIL-10) encoded by the native transgene constructs was identical to the native human IL-10. After purification and thrombin cleavage, the prIL-10 encoded by the tagged construct differs in only its two terminal amino acids from the native hIL-10.

15

Gene constructs are transformed into *N. tabacum* cv. 81V9-4 low alkaloid tobacco strains according to the method described by Miki et al. (1998). Transformed and regenerated plants are initially selected for kanamycin resistance. Regenerated plants are analyzed for expression of the IL10 protein by solid phase sandwich Enzyme-Linked ImmunoSorbent Assay (ELISA). Briefly, the JES3-9D7 anti-IL-10 mAB (PharMingen) is used as a capture antibody and is paired with the biotinylated JES3-12G8 anti-IL-10 mAB (PharMingen) as the detecting antibody. Recombinant human IL-10 (PharMingen) is used as standard.

20

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Total soluble protein extracts are prepared from 1 gram of young leaf tissue by homogenizing the tissue in 1 ml of a buffer containing phosphate buffered saline-Tween (PBS-T), 2% polyvinylpolypyrrolidone (PVPP), 1mM ethylenediaminetetraacetic acid (EDTA), 1mM phenylmethyl sulfonyl fluoride (PMSF) and 1 µg/ml Leupeptin. The homogenate is then collected in a tube and spun at 10,000 g for 15 min in a refrigerated

30

centrifuge. The supernatant is collected and used directly in the ELISA. The results are presented in Figure 7.

Eighteen transgenic plants are regenerated from the native construct containing only a short stretch of 3' UTR (called E6 and E15 in Figure 7), and nineteen plants are regenerated from the native construct containing the entire 3' UTR (called F10 in Figure 7). All of those and the first two plants regenerated from the construct containing the His tag and KDEL ER retention signal (called G7 in Figure 7) are tested for presence of IL-10 protein by ELISA.

A variable level of plant recombinant IL-10 (prIL-10) is observed in the regenerated plants. There were no marked differences between plants transformed with the full-length hIL-10 cDNA and those transformed with 3' truncated cDNA. However, the highest level of prIL-10 is obtained in one of the two plants transformed with the construct containing the ER retention signal, G7-2.

Following the detection of IL-10 transcripts and protein in the transgenic plants crude protein extracts containing prIL-10 the demonstration of bioactivity of human IL-10 is determined *in vitro* by its effect on T cell proliferation in response to lectins (PHA) as well as in standard mixed lymphocyte responses (MLR). Plant extract or purified IL-10 from plant extract is added to media containing human lymphocytes, at a concentration ranging from 1-100 ngm/ml. Proliferation is determined in PHA cultures and mixed lymphocyte reaction cultures at days 3-6 by standard ³H- thymidine incorporation proliferation assays.

Surface expression of MHC class II proteins is determined on human lymphocytes activated by either PHA or ConA in the presence of increasing concentrations of IL-10. And compared to control plant extracts.

Peripheral blood lymphocytes are stimulated with LPS (1-10 μ /ml) for 24-48 hours, and levels of cytokines determined by ELISA or bioassay. Inhibition is determined as well, at the RNA level, by Northern blot analysis.

5 IL-10 is beneficial in prevention of lethal endotoxemia. Following appropriate approval the effect of IL-10 purified from plant extract on endotoxic injury in BALB/c mice is tested. *E. coli* is administered interperitoneally or intravenously in the presence of various amounts of recombinant murine IL 10. Plant extract is given to mice orally in addition to purified IL-10 from plants given intravenously. Outcomes are determined by
10 survival of mice, which is essentially zero percent in untreated mice. Appropriate controls include PBS treated mice as well as mice given standard animal chow.

IL-10 deficient mice develop bowel inflammation and lesions resembling inflammatory bowel disease, which is improved by systemic IL-10. To test whether oral
15 plant IL-10 can prevent bowel disease in an IL-10 deficient mouse model of IBD, transgenic plant material is administered to IL-10 null mice daily from age 4 weeks and mice are assessed for the presence of bowel lesions from age 8 to 30 weeks by weight gain and histology of tissue.

20 **Example 4: Alkaloid levels in leaf tissue of the non-food bioreactor**

In order to establish concentrations of tobacco alkaloids in normal and non-food bioreactor plants a field trial was conducted during the summer of 1997. Transplants of Delgold, a tobacco cultivar with normal alkaloid concentrations and 81V-9 the non-food
25 bioreactor genotype used for this present invention were started in mid April in a cold frame greenhouse located at the Delhi Research Station in Ontario Canada. The two cultivars were transplanted into a 1.2 x 2 m plots at a density of approximately 133,000 plants per hectare. The trial employed a randomized complete design with four replications and was fertilized with 60 kg nitrogen per hectare. The trial was harvested
30 40 days after planting, the tissue was roughly chopped, quick frozen in liquid nitrogen and held at -70 °C until ready for analysis. The tissue was then lyophilized and tobacco

alkaloid concentrations in the tissue determined by gas chromatography. For this analysis approximately 1 gram of lyophilized tobacco tissue was ground to pass a 20 mesh screen and was then extracted with dichloromethane and aqueous sodium hydroxide by shaking for 10 min on a wrist action shaker. The dichloromethane layer was filtered and collected for alkaloid analysis. The internal standard method was used with anethole being that standard. All gas chromatographic analyses were performed on an Hewlett Packard 5890 series II using a DB5 60m x.25mm i.d. column with splitless injection and a 2 µl sample volume. Total tobacco alkaloid concentrations were found to be 35 fold lower in the non-food bioreactor than the normal tobacco cultivar, nicotine concentration had a similar pattern to the total and accounted for between 92 and 99 % of the total (Table 1). The levels of anabasine were similar in the two lines and those of anatabine higher in the non-food bioreactor than Delgold.

Table 1.

Concentration (mg/g) of the various tobacco alkaloids found in the leaves of normal tobacco and in the non-food bioreactor.

Cultivar	Nicotine	Myosmine	Anabasine	Anatabine	Total	% of total nicotine	% of total alkaloid
----- mg/g -----							
Delgold	7.892a*	0.000a	0.013a	0.000a	7.923a	100	100
81V-9	0.209b	0.000a	0.018a	0.020b	0.227b	2.6	0.28

*means followed by a different letter are significantly different from each other at P=0.05

All scientific publications and patent documents are incorporated herein by reference.

The present invention has been described with regard to preferred embodiments. However, it will be obvious to persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention as described in the following claims.

References

- Abrams, J.S., M.G. Roncarolo, H. Yasel, U. Anderson, G. Gleich, J. Silver (1992) Strategies of anti-cytokine monoclonal antibody development. *Immunol. Rev.* 127, 5-24.
- Bradford M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* 72, 248-254.
- Burnette W.H. (1981) Western blotting: electrophoretic transfer of proteins from SDS-polyacrylamide gels to unmodified nitrocellulose and radio-graphic detections with antibody and radioiodinated protein A. *Anal. Biochem.* 112, 195-203.
- Chaplin J.F. (1977) Breeding for varying levels of nicotine in tobacco. In: *Proc Amer Chem Soc*, 173rd meeting, New Orleans.
- Cornelissen B.J.C, R.A.M Hooft van Huijsduijnen, L.D.Van Loon & J.F.Bol (1986) Molecular characterization of messenger mRNAs for "pathogenesis-related" proteins 1a, 1b and 1c, induced by TMV infection of tobacco. *EMBO J.* 5, 37-40.
- Denecke J., J.Botterman & R.Deblaere (1990) Protein secretion in plant cells can occur via a default pathway. *Plant Cell* 2, 51-59.
- Fourney R.M., J.Miyakoshi, R.S.Day III & M.C.Paterson (1988) Northern blotting: efficient RNA staining and transfer. *Focus* 10:1, 5-7.
- Fiocchi C. (1993) Cytokines and animal models: a combined path to inflammatory bowel disease pathogenesis. *Gastroenterology* 104:1202-1219.
- Frisch, D. A., Harris-Haller, L.W., Yokubaitis, N.T., Thomas, T.L., Hardin, S.H., and Hall, T.C. (1995). Complete sequence of the binary vector Bin 19. *Plant Mol. Biol.* 27, 405-409.
- Gengenheimer P (1990) Preparation of Extracts from Plants. *Methods of Enzymology* 182, 184-185.
- Haq T.A., H.S. Mason, J.D. Clements, C.J. Artzen (1995) Oral immunization with a recombinant bacterial antigen produced in plants. *Science* 268, 714-716.
- Hiatt A., R.Cafferkey & K.Bowdish (1989) Production of antibodies in transgenic plants. *Nature* 342, 76-78.

- Horsch R.B., J.Fry, N.Hofmann, J.Neidermeyer, S.G.Rogers & R.T.Fraley. (1988) Leaf disc transformation. Plant Molecular Biology Manual A5, 1-9. Ed. S.B.Gelvin, R.A.Schilperoort, D.P.S.Verma. Kluwer Academic Publishers. Dordrecht/Boston/London.
- Kay R., A.Chan, M.Daly & J.McPherson (1987) Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science 236, 1299-1302.
- Kuhn R. LohlerJ, Rennick D, Rajewsky K, Muller W (1993) Interleukin-10 deficient mice develop chronic enterocolitis. Cell 75:263-274.
- Kunkel T.A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA 82, 488-492.
- Lehrach H., D.Diamond, J.M.Wozney & H. Boedtker. (1977) RNA molecular weight determinations by gel electrophoresis underdenaturing conditions; a critical reexamination. Biochemistry 16, 4743.
- Ma J.K.C., M. Hein (1995) Immunotherapeutic potential of antibodies produced in plants. TibTech 13, 522-527.
- Mason H.S., J.M. Ball, J-J. Shi, X. Jiang, M.K. Estes, C.J. Arntzen (1996) Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice. Proc. Nat. Acad. Sci. 93, 5335-5340.
- Miki, B.L.A., McHugh, S.G., Labbe, H., Ouellet, T., Tolman, J.H., and Brandle, J.E. (1998) Transgenic tobacco: gene expression and applications. Biotechnology in Agriculture and Forestry (in press)
- Montanari L., P. Fantozzi, S. Pedone (1993) Tobacco fraction 1 (F1P utilization of oral feeding and enteral feeding of patients. 1 Heavy Metal evaluation. Food SAc. Tech. 26, 259-263.
- Otsuka T., D. Vallaret, T. Yokota, Y. Takebe, F. Lee, N. Arai, K. Arai (1987) Structural analysis of the mouse chromosomal gene encoding interleukin-4 which expresses B cell, T cell and mast cell stimulating activities. Nucl. Acid Res. 15, 333-334
- Palmiter R.D. (1974) Magnesium precipitation of ribonucleoprotein complexes: Expedient techniques for the isolation of undegraded polysomes and messenger ribonucleic acid. Biochemistry 13, 3606-3615.
- Perlak FJ, Fuchs RL, Dean DA, McPherson SL, Fischhoff DA (1991) Modification of the coding sequence enhances plant expression of insect control protein genes. Proc Natl Acad Sci 88:3324-3328.

- Rapoport M.J., D. Zipris, A.H. Lazarus, A. Jaramillo, D.V. Serreze, E.H. Leiter, P. Cyopick, J.S. Danska, T.L. Delovitch (1993) IL-4 reverses thymic T cell proliferative unresponsiveness and prevents diabetes in NOD mice. *J. Exp. Med.* 178, 87-99.
- Richards K., H.Guilley, G.Jonard & G.Keith (1977) Leader sequence of 71 nucleotides devoid of G in tobacco mosaic virus RNA. *Nature* 267, 548-550.
- Richards K., H.Guilley, G.Jonard & L.Hirth (1978) Nucleotide sequence at the 5' extremity of tobacco mosaic virus RNA. *Eur. J. Biochem.* 84, 513-519.
- Schouten A et al. (1966) The C-terminal KDEL sequence increases the expression level of a single-chain antibody designed to be targeted to both the cytosol and the secretory pathway in transgenic tobacco. *Plant Molec. Biol.* 30, 781-793.
- Sleat D.E., R.Jull, P.C.Turner & T.M.A.Wilson (1988) On the mechanism of translational enhancement by the 5'-leader sequence of tobacco mosaic virus RNA. *Eur. J. Biochem.* 175, 75-86.
- Sijmons P.C., B.M.M.Dekker, B.Schrammeijer, T.C.Verwoerd, P.J.M.van den Elzen & A.Hoekema (1990) Production of correctly processed human serum albumin in transgenic plants. *Bio/Technology* 8, 217-221.
- Vieira, P., de Waal-Maleyfy, R., Dang, M.N., Johnson, K.E., Kastelein, R., Fiorentino, D.F., deVries, J.E., Roncarolo, M.G., Mosmann, T.R., and Moore, K.W. (1991) Isolation and expression of human cytokine synthesis inhibitory factor cDNA clones: homology to Epstein-Barr virus open reading frame BCRF1. *Proc. Natl. Acad. Sci. U.S.A.* 88, 1172-1176.
- von Heijne G. (1986) A new method for predicting signal cleavage sites. *Nucl. Acids Res.* 14, 4683-4690.
- Woodleif W.G., J.F. Chaplin, C.R. Campbell, D.W. DeJong (1981) Effect of variety and harvest treatments on protein yield of close grown tobacco *Tobacco Sci.* 25, 83-86.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OF PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A method for the preparation of a protein of interest for oral administration, comprising:
 - i) transforming a non-food crop plant with a vector containing a gene of interest operatively linked with 5' and 3' regulatory regions;
 - ii) selecting transformed non-food crop plants that contain the protein of interest
 - iii) growing and harvesting selected non-food crop plants; and
 - iv) obtaining plant tissues that comprise the protein of interest;wherein said non-food crop plant is characterized as being non-toxic, non-addictive, palatable, and requiring minimal or no processing prior to oral administration.
2. The method of claim 1 wherein the non-food plant is characterized in having a low alkaloid level.
3. The method of claim 2 wherein the non-food crop plant is a tobacco plant characterized as having a low nicotine level.
4. The method of claim 3 wherein the protein of interest is a pharmaceutically active protein.
5. The method of claim 4 wherein the pharmaceutically active protein is an interleukin or derivatives or combinations thereof.

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6. The method of claim 4 wherein the 5' and 3' regulatory regions to ensure expression of the gene of interest include ubiquitous or tissue specific promoters.
7. The method of claim 6 wherein the appropriate regulatory regions include an endoplasmic reticulum retention motif.
8. The method of claim 7 wherein the appropriate regulatory regions include a plant signal sequence.
9. A protein composition prepared by the method of claim 1 comprising a pharmaceutically active protein and soluble plant components.
10. The composition of claim 9 wherein the pharmaceutically active transgenic protein of interest is an interleukin, or derivatives or combinations thereof.
11. The composition of claim 9 wherein the non-food crop is tobacco.
12. The composition of claim 9 wherein the tissue is leaf.
13. A method for the treatment of a medical ailment comprising orally administering an effective amount of the composition of claim 10 to a patient in need thereof.
14. The method of claim 13 wherein the medical ailment is selected from the group consisting of diabetes, conditions related to blood clotting, medical conditions requiring the stimulation of progenitor cells to monocytes/macrophages, or viral infections.
15. A method for preparing a pharmacologically active protein within a non-food crop plant comprising;

- 36 -

- i) selecting a non-transformed tobacco plant comprising from about 0.2% to about 20% of the total alkaloid, and characterized as being non-toxic, non-addictive, and palatable;
- ii) transforming said non-transformed tobacco plant with a vector comprising a gene capable of encoding said pharmacologically active protein and 5' and 3' regulatory regions to ensure expression of the gene within said non-transformed tobacco plant to produce a transformed plant; and
- iii) obtaining said pharmacologically active protein from said transformed plant.

16 A method for the preparation of IL-10 comprising;

- i) transforming a plant with a vector comprising a gene encoding IL-10, operatively linked with 5' and 3' regulatory regions;
- ii) selecting transformed plants that contain IL-10;
- iii) growing and harvesting selected plants; and
- iv) extracting IL-10 from said selected plants.

17 The method of claim 16 wherein said IL-10 is human IL-10

18 The method of claim 16 wherein said vector also comprises sequences that enhance expression of said IL-10.

19 The method of claim 18 wherein said sequences that enhance expression include an ER retention signal.

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20. The method of claim 16 wherein said vector encodes an affinity tag.
21. The method of claim 16 wherein said vector also encodes a proteolytic cleavage site

1/6

✓

4363

114365

Figure 1

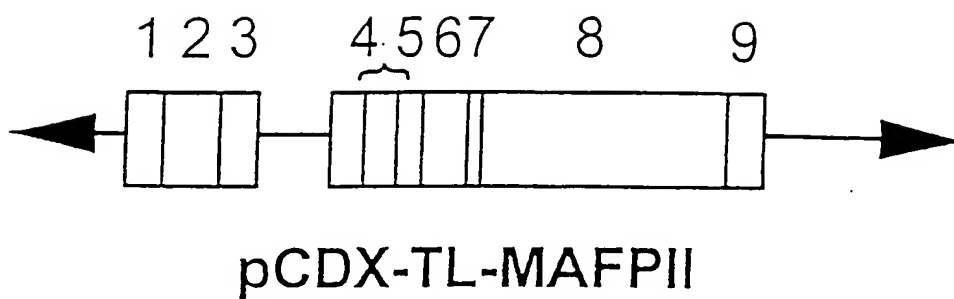


Figure 2

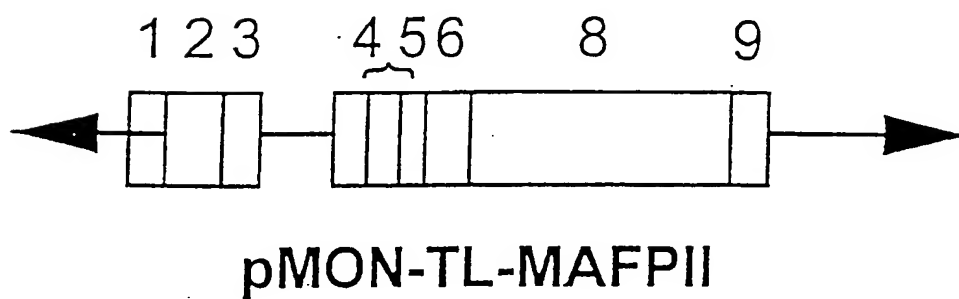


Figure 3

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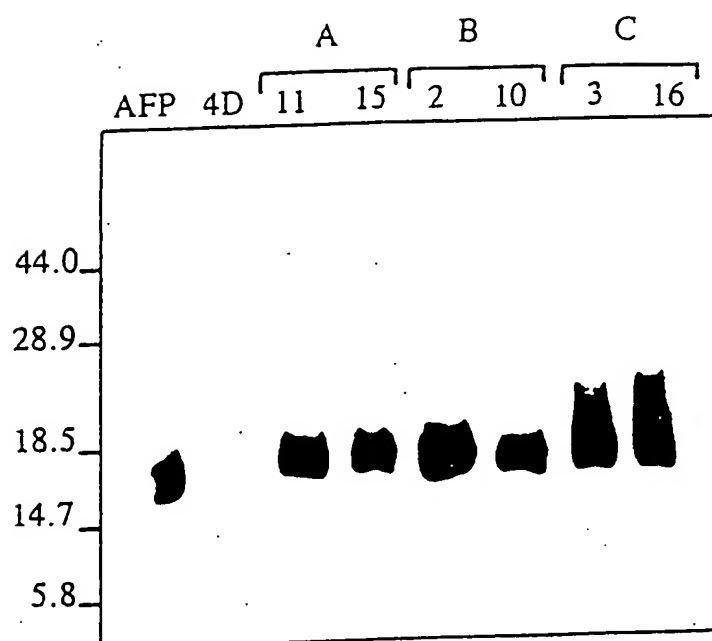


FIGURE 4

hIL10-5'-BamHI

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Signal peptide

GGATCCACAAGACGACTTGCAAAAGAAGG
1 aaaccacaagacgacttgcaaaagaaggcatgcacagctcagcactgtctgttgcctg
M H S S A L L C C L

61 gtccctcctgactggggtgagggccagcccaggccagggcacccagtctgagaacagctgc
V L L T G V R A S P G Q G T Q S E N S C

121 acccacttcccaggcaacctgcctaacatgcttcgagatctccgagatgccttcagcaga
T H F P G N L P N M L R D L R D A F S R

181 gtgaagacttttctttcaaataagagatcagctggacaacttgttgtaaaggagtccttg
V K T F F Q M K D Q L D N L L L K E S L

241 ctggaggactttaagggttacctgggttgccaagccttgtctgagatgatccagttttac
L E D F K G Y L G C Q A L S E M I Q F Y

301 ctggaggaggtgatgccccaagctgagaaccaagaccagacatcaaggcgcatgtgaac
L E E V M P Q A E N Q D P D I K A H V N

361 tccctgggggagaacctgaagacctcagggctgagggtacggcgctgtcatcgatttctt
S L G E N L K T L R L R L R R C H R F L

421 ccctgtgaaaaacaagagcaaggccgtggagcaggtgaagaatgcctttaataagctccaa
P C E N K S K A V E Q V K N A F N K L Q

481 gagaaggcatctacaaagccatgagtgtgtttgacatcttcatcaactacatagaagcc
E K G I Y K A M S E F D I F I N Y I E A

hIL-10-His-KDEL

M T M K I R N L V P R G S S S G H H H
TACTGTACTTCTATGCATTGGAACAGGGTGCGCCAAAGAAGAAGACCAGTAGTAGTA

541 tacatgacaatgaagatacgaactgagacatcagggtggcgactctatagactctagga
Y M T M K I R N *

H H H K D E L *

G TAGTAGTGTTCTCTGCTCGAGACTTAAGG

601 cataaattagaggtctccaaaatcggatct ggggctctgg gatagctgac ccagccccctt
GG AGTTGGGGGT

661 gagaaacctt attgtacctc tcttatagaa tatattattac ctctgatacc tcaaccccc
GG AGTTGGGGGT

721 tttctattta ttactgagc ttctctgtga acgatttaga aagaagccca atattataat
AAAGATAAAT AACTTAAGG
hIL10-3'-EcoRI

781 ttttttcaat atttattatt ttcacctgtt ttaagctgt ttccataggg tgacacacta
841 tggattttga gtgttttaag ataaattata agttacataa gggaggaaaa aaaatgttct
901 ttggggagcc aacagaagct tccattccaa gcctgaccac gctttctagc tgttgagctg
961 ttttccctga cctccctcta atttatcttg tctctgggct tggggcttcc taactgctac
1021 aaatactctt aggaagagaa accaggggagc ccctttgatg attaattcac cttccagtgt
1081 ctcgaggaggga ttcccctaac ctcatccccc aaccacttca ttcttgaaag ctgtggccag
1141 cttgttattt ataacaacct aaatttggtt ctaggccggg cgcggtggct cacgcctgta
1201 atcccagcac tttgggaggc tgaggcgggt ggatcacttg aggctcaggag ttccctaacca
1261 gcctggtcaa catggtgaaa ccccgctctt actaaaaata caaaaattag ccgggcatgg
1321 tggcgcgcac ctgtaatccc agctacttgg gaggtctgag caagagaatt gcttgaacco
1381 aggagatgga agttgcagtg agctgatatc atgcccctgt actccagcct ggggtgacaga
1441 gcaagactct gtctcaaaaa aataaaaaata aaaataaatt tggttctaata agaactcagt
1501 ttttaactaga atttattcaa ttccctctgg aatgtttacat tgtttgtctg tcttcataga
1561 agattttaat tttgaataaa taaatgtatc ttattcacat c

FIGURE 5

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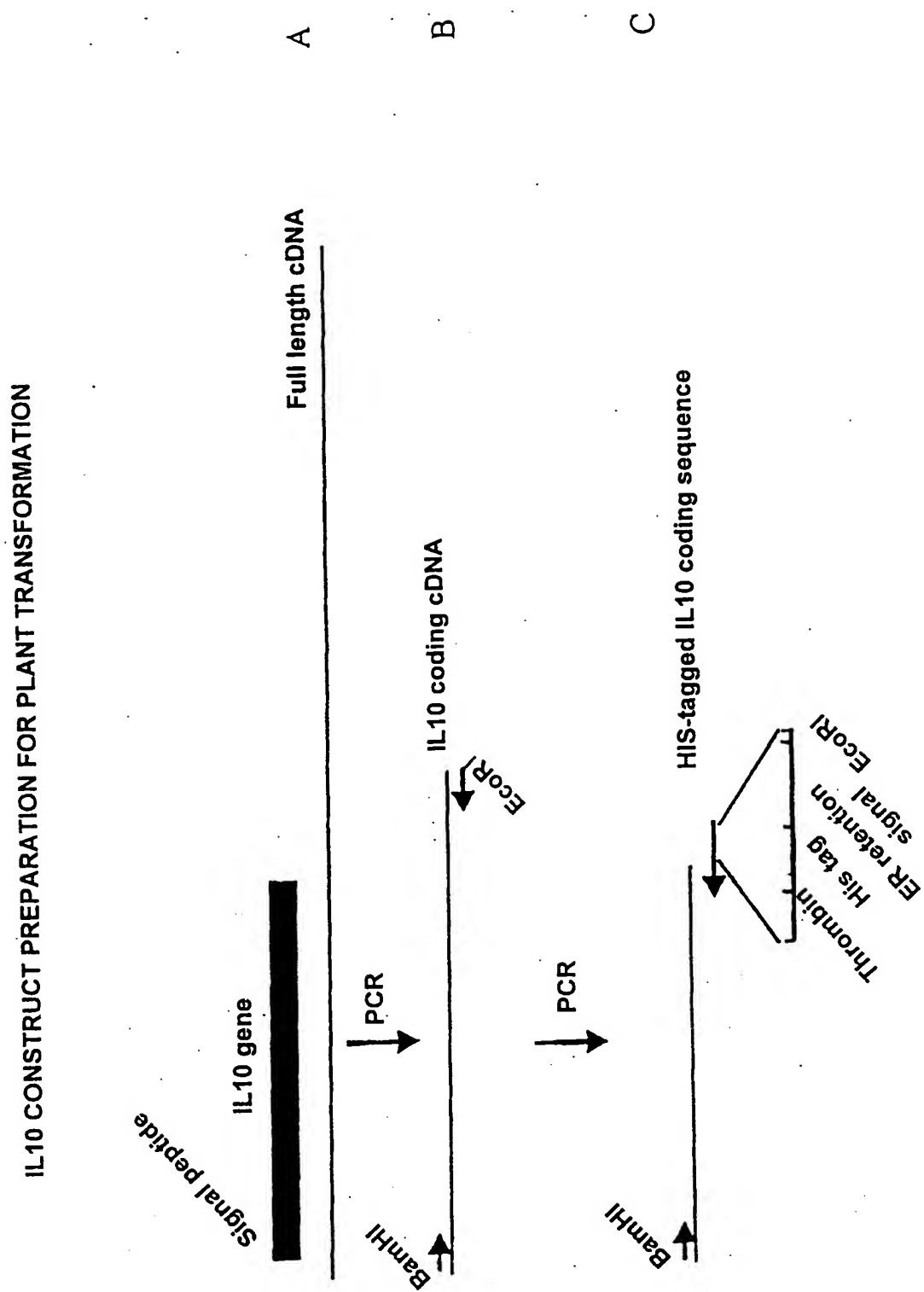
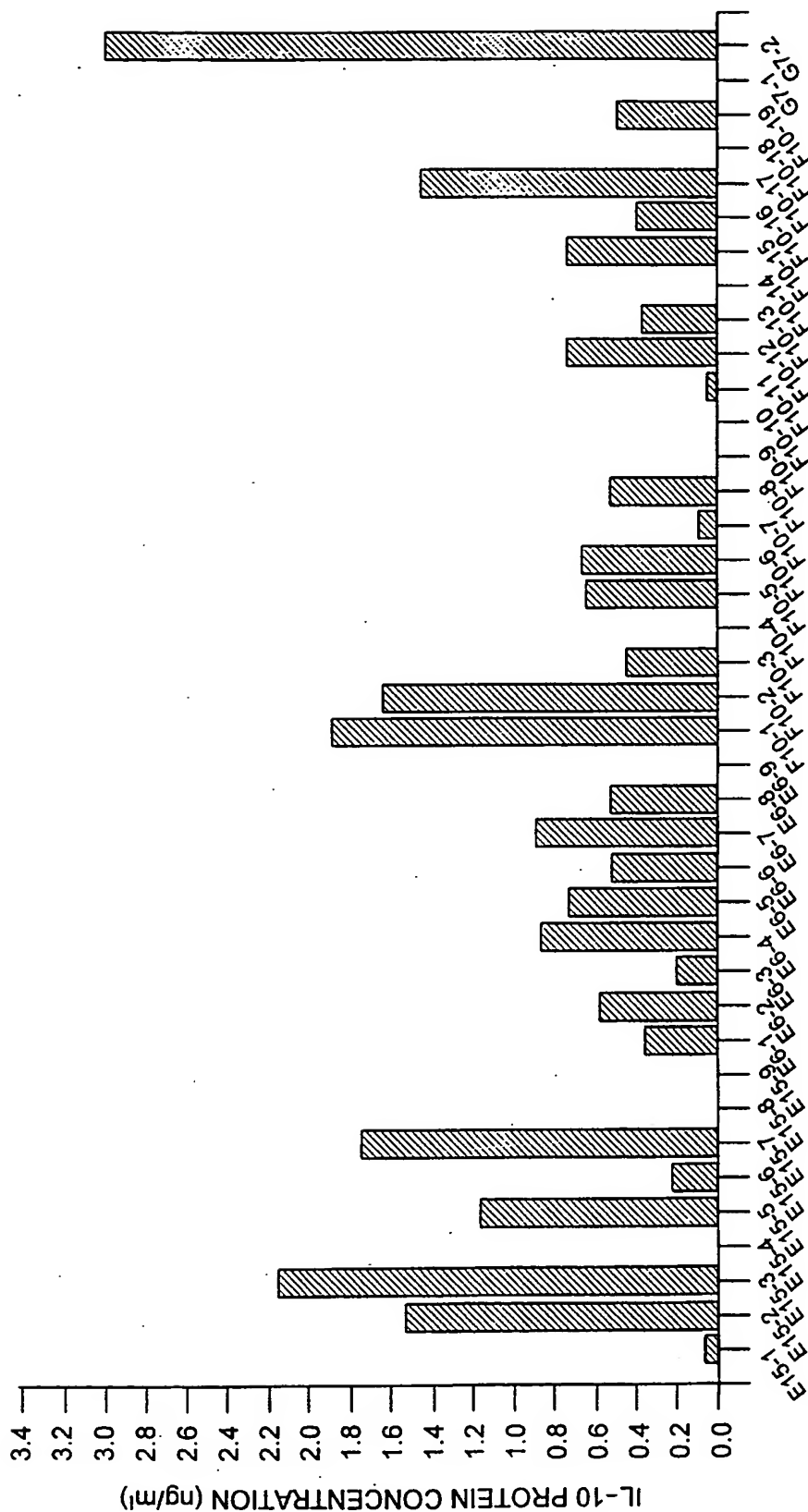


FIGURE 6

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REGENERATED PLANTS

FIGURE 7

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Jim Brandle
- (A) NAME: Peter Davis
- (A) NAME: Kimberly Kenward
- (A) NAME: Rima Mensassa
- (A) NAME: Tony Jevnikar
- (A) NAME: Terry Delovitch

(ii) TITLE OF INVENTION: Non-Food Crop Plant Bioreactor

(iii) NUMBER OF SEQUENCES: 12

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Tobacco mosaic virus

(vii) IMMEDIATE SOURCE:

- (B) CLONE: 1091

(viii) POSITION IN GENOME:

- (C) UNITS: bp

(x) PUBLICATION INFORMATION:

- (A) AUTHORS: Richards, K
Guilley, H
Jonard, G
Keith, G
- (B) TITLE: Leader sequence of 71 nucleotides devoid of G
in tobacco mosaic virus RNA

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- (C) JOURNAL: Nature
- (D) VOLUME: 267
- (F) PAGES: 548-550
- (G) DATE: 1977
- (x) PUBLICATION INFORMATION:
 - (A) AUTHORS: Richards, K
Guilley, H
Jonard, G
Hirth, L
 - (B) TITLE: Nucleotide sequence at the 5' extremity of tobacco mosaic virus RNA
 - (C) JOURNAL: Eur. J. Biochem.
 - (D) VOLUME: 84
 - (F) PAGES: 513-519
 - (G) DATE: 1978

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GATCCTCGAG TATTTTACA

20

(2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 58 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "oligonucleotide"
- (vii) IMMEDIATE SOURCE:
 - (B) CLONE: 1309

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

ACAATTACCA ACAACAACAA ACAACAAACA ACATTACAAT TACTATTTAC AATTACAA

58

(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 49 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "oligonucleotide"
- (iii) HYPOTHETICAL: NO

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(vii) IMMEDIATE SOURCE:
(B) CLONE: 4361

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ACAATTACCA ACAACAACAA ACAACAAACA ACATTACAAT TACTATTTA

49

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 74 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(vii) IMMEDIATE SOURCE:
(B) CLONE: 1092

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TTGTAATTGT AAATAGTAAT TGTAATGTTG TTTGTTGTTT GTTGTTGTTG GTAATTGTTG

60

TAAAAATACT CGAG

74

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(vii) IMMEDIATE SOURCE:
(B) CLONE: 4362

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CAATTACAAT GGGATTTTTT CTCTTTTCAC AAATGCCAAG CTTTTTCTT G

51

(2) INFORMATION FOR SEQ ID NO: 6:

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(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 46 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "oligonucleotide"

(vii) IMMEDIATE SOURCE:

- (B) CLONE: 4363

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TCAGTACTCT TCTCTTATTC CTGATCATAT CTCACTCTTC GCATGC

46

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:

- (B) CLONE: PR signal peptide cassette

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Met	Gly	Phe	Phe	Leu	Phe	Ser	Gln	Met	Pro	Ser	Phe	Phe	Leu	Val	Ser
1				5				10					15		

Thr	Leu	Leu	Leu	Phe	Leu	Ile	Ile	Ser	His	Ser	Ser	His	Ala
			20				25					30	

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 56 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "oligonucleotide"

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(vii) IMMEDIATE SOURCE:

(B) CLONE: 4364

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GAGAAGAGTA CTGACAAGAA AAAAGCTTGG CATTGTGAA AAGAGAAAAA ATCCCA

56

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 34 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "oligonucleotide"

(vii) IMMEDIATE SOURCE:

(B) CLONE: 4365

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TAGCATGCGA AGAGTGAGAT ATGATCAGGA ATAA

34

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "oligonucleotide"

(vii) IMMEDIATE SOURCE:

(A) LIBRARY: 3339

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

TATTTTTTAC AACAAATTACC AACAAAC

26

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(vii) IMMEDIATE SOURCE:
(B) CLONE: 4708

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CAGCAGTCAT CTGCATACAG CAC

23

(2) INFORMATION FOR SEQ ID NO: 12:

(i). SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1645 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:
(B) CLONE: hIL-10-His-KDEL

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

AAACCACAAG ACAGACTTGC AAAAGAAGGC ATGCACAGCT CAGCACTGCT CTGTTGCCTG	60
GTCCTCCTGA CTGGGGTGAG GGCCAGCCCA GGCCAGGGCA CCCAGTCTGA GAACAGCTGC	120
ACCCACTTCC CAGGCAACCT GCCTAACATG CTTCGAGATC TCCGAGATGC CTTCAGCAGA	180
GTGAAGACTT TCTTTCAAAT GAAGGATCAG CTGGACAACT TGTTGTTAAA GGAGTCCTTG	240
CTGGAGGACT TTAAGGGTTA CCTGGGTGTC CAAGCCTTGT CTGAGATGAT CCAGTTTAC	300
CTGGAGGAGG TGATGCCCA AGCTGAGAAC CAAGACCCAG ACATCAAGGC GCATGTGAAC	360
TCCCTGGGGG AGAACCTGAA GACCCTCAGG CTGAGGCTAC GCGCTGTCA TCGATTCTT	420
CCCTGTGAAA ACAAGAGCAA GGCCGTGGAG CAGGTGAAGA ATGCCTTTAA TAAGCTCCAA	480
GAGAAAGGCA TCTACAAAGC CATGAGTGAG TTTGACATCT TCATCAACTA CATAGAAGCC	540
TACATGACAA TGAAGATACG AACTGAGAC ATCAGGGTGG CGACTCTATA GACTCTAGGA	600

7/7

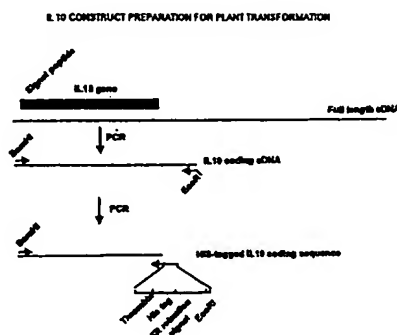
CATAAATTAG AGGTCTCCAA AATCGGATCT GGGGCTCTGG GATAGCTGAC CCAGCCCCCTT	660
GAGAAACCTT ATTGTACCTC TCTTATAGAA TATTTATTAC CTCTGATACC TCAACCCCCA	720
TTTCTATTTA TTTACTGAGC TTCTCTGTGA ACGATTTAGA AAGAAGCCCA ATATTATAAT	780
TTTTTTCAAT ATTTATTATT TTCACCTGTT TTAAAGCTGT TTCCATAGGG TGACACACTA	840
TGGTATTTGA GTGTTTAAAG ATAAATTATA AGTTACATAA GGGAGGAAAA AAAATGTTCT	900
TTGGGGAGCC AACAGAAGCT TCCATTCCAA GCCTGACCAC GCTTTCTAGC TGTGAGCTG	960
TTTTCCCTGA CCTCCCTCTA ATTTATCTTG TCTCTGGGCT TGGGGCTTCC TAACTGCTAC	1020
AAATACTCTT AGGAAGAGAA ACCAGGGAGC CCCTTTGATG ATTAATTCAC CTTCCAGTGT	1080
CTCGGAGGGA TTCCCCTAAC CTCATTCCCC AACCACCTCA TTCTTGAAAG CTGTGGCCAG	1140
CTTGTTATTT ATAACAACCT AAATTGTT CTAGGCCGGG CGCGGTGGCT CACGCCTGTA	1200
ATCCCAGCAC TTTGGGAGGC TGAGGCGGGT GGATCACTTG AGGTCAGGAG TTCCTAACCA	1260
GCCTGGTCAA CATGGTGAAA CCCCCTCTCT ACTAAAAATA CAAAAATTAG CCGGGCATGG	1320
TGGCGCGCAC CTGTAATCCC AGCTACTTGG GAGGCTGAGG CAAGAGAATT GCTTGAACCC	1380
AGGAGATGGA AGTTGCAGTG AGCTGATATC ATGCCCTGT ACTCCAGCCT GGGTGACAGA	1440
GCAAGACTCT GTCTCAAAAA AATAAAAATA AAAATAAATT TGGTTCTAAT AGAACTCAGT	1500
TTTAACTAGA ATTTATTCAA TTCCTCTGGG AATGTTACAT TGTTTGTCTG TCTTCATAGC	1560
AGATTTTAAT TTTGAATAAA TAAATGTATC TTATTCACAT CAAAAAAAAA AAAAAAAAAA	1620
CTCGAGGGGG GGCCGGTACC CAATG	1645



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/82, 15/12, A61K 38/17, 35/78		A3	(11) International Publication Number: WO 99/67401
			(43) International Publication Date: 29 December 1999 (29.12.99)
(21) International Application Number: PCT/CA99/00577		(74) Agents: SECHLEY, Konrad, A. et al.; Gowling, Strathy & Henderson, Suite 2600, 160 Elgin Street, Ottawa, Ontario K1P 1C3 (CA).	
(22) International Filing Date: 21 June 1999 (21.06.99)			
(30) Priority Data: 09/102,050 22 June 1998 (22.06.98) US		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(71) Applicants: HER MAJESTY THE QUEEN IN RIGHT OF CANADA as represented by THE MINISTER OF AGRICULTURE AND AGRI-FOOD CANADA [CA/CA]; Pest Management Research Centre, 1391 Sanford Street, London, Ontario N5V 4T3 (CA). QUEEN'S UNIVERSITY [CA/CA]; Kingston, Ontario K7L 3N6 (CA). THE JOHN P. ROBERTS RESEARCH INSTITUTE [CA/CA]; 100 Perth Drive, London, Ontario N6A 5K8 (CA). LONDON HEALTH SCIENCES CENTER [CA/CA]; 339 Windermere Road, London, Ontario N6A 5A5 (CA).		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
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(54) Title: NON-FOOD CROP PLANT BIOREACTOR



(57) Abstract

A novel method for the production of transgenic proteins of interest suitable for oral administration is disclosed. The method involves the preparation of a protein of interest suitable for oral administration within a non-food crop plant comprising, transforming the non-food crop plant with a suitable vector containing a gene of interest and appropriate regulatory regions to ensure expression of the gene of interest within the non-food crop plant, such that the non-food crop plant is characterized as being non-toxic, non-addictive, palatable, and requiring minimal or no processing prior to oral administration. An example of a non-food crop plant is low alkaloid tobacco. Proteins of interest may include pharmaceutically active proteins such as growth regulators, insulin, interferons, interleukins, growth hormone, erythropoietin, G-CSF, GM-CSF, hPG-CSF, M-CSF, Factor VIII, Factor IX, tPA, antibodies, antigens and combinations and/or derivatives thereof.

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INTERNATIONAL SEARCH REPORT

Inter Application No

PCT/CA 99/00577

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/82 C12N15/12 A61K38/17 A61K35/78

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
L, X	CA 2 188 220 A (UNIV KINGSTON ;MINI OF AGRICULTURE AND AGRI F (CA)) 18 April 1998 (1998-04-18)	1-15
Y	example 2	16-21
Y	WO 93 03161 A (DONSON JON ;DAWSON WILLIAM O (US); GRANTHAM GEORGE L (US); TURPEN) 18 February 1993 (1993-02-18) claims 26,33,38,40,41	16-21
T	SPURGEON D.: "Tobacco could be used to produce interleukin 10" BRITISH MEDICAL JOURNAL, vol. 319, no. 7203, 17 July 1999 (1999-07-17), pages 143-143, XP002127522 the whole document	1-21

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

18 January 2000

Date of mailing of the international search report

01/02/2000

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Authorized officer

Schönwasser, D

INTERNATIONAL SEARCH REPORT

national application No.

PCT/CA 99/ 00577

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 13 and 14
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 13 and 14
are directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

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- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern. Application No

PCT/CA 99/00577

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
CA 2188220 A	18-04-1998	NONE	
WO 9303161 A	18-02-1993	US 5529909 A	25-06-1996
		US 5589367 A	31-12-1996
		US 5922602 A	13-07-1999
		US 5977438 A	02-11-1999
		US 5889190 A	30-03-1999
		US 5866785 A	02-02-1999
		US 5316931 A	31-05-1994
		AU 683412 B	13-11-1997
		AU 3351193 A	02-03-1993
		CA 2114636 A	18-02-1993
		EP 0596979 A	18-05-1994
		JP 7503361 T	13-04-1995

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